

The Urinary Cotinine and Serum 25 Hydroxyvitamin D Levels in Male Smokers

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Objective: To assess urinary cotinine and the effects of smoking on 25 (OH) D levels in 67 male smokers.

Material and Method: Urine and blood specimens were analyzed for cotinine and serum 25 hydroxyvitamin D (25 (OH) D) concentrations by high performance liquid chromatography (HPLC) and chemiluminescent immunoassay, respectively. The accuracy, precision and detection limit of the HPLC method were also tested.

Results: The detection limit of urinary cotinine was 0.02 µg/ml. The recoveries of cotinine concentrations of 0.15-2.0 µg/ml were greater than 95%. Only 23.9% of smokers had sufficient levels of serum 25 (OH) D at least 30 ng/ml. The smokers were divided into dairy co-operative smokers and other smokers. The average urinary cotinine concentrations of 1,421.42 and 1,866.52 µg/g creatinine were not significant different in dairy co-operative smokers and other smokers whereas the average 25 (OH) D of 29.09 and 22.65 ng/ml, respectively, were significantly different at p-value of < 0.001.

Conclusion: The 42.86% and 10.26% of the dairy co-operative smokers and other smokers had sufficient serum 25 (OH) D levels to prevent osteoporosis.

Keywords: Urinary cotinine, Serum 25 hydroxyvitamin D, Smokers, High-performance liquid chromatography

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The toxic chemicals in cigarettes directly impacting upon vitamin D metabolism are unclear. Vitamin D plays an important role in the health of bones. Vitamin D deficiency in adults is known to precipitate or exacerbate osteopenia and osteoporosis, can cause osteomalacia and muscle weakness and increases the risk of fracture⁽¹⁾. A serum level of 25 hydroxy vitamin D (25 (OH) D) is a biomarker to assess vitamin D status. In the previous study, it was found that there were significant differences in BMD (bone mineral density) between heavy smokers (more than 20 cigarettes/day) and nonsmokers in all skeletal sites⁽¹⁾. However, most studies of the relationship between smoking and osteoporosis have been carried out in postmenopausal women or elderly men after having had a long-time

exposure to the toxic agent in cigarettes^(2,3). There is little information regarding the effect of smoking on bones in young people. The number of Thai males who smoked daily was 3,600,977 among the age group from 25 to 40 years and was 3,256,445 among the age group from 41 to 59 years⁽⁴⁾. To measure the exposure of nicotine in smokers, cotinine is widely used as biomarkers⁽⁵⁾. Cotinine is mostly determined in urine, a biological medium easy to obtain. Cotinine is appropriate because of the longer urinary half-life of cotinine compared with nicotine (20 vs. 2 h)⁽⁶⁾. The objective of the present study was to assess urinary cotinine and the effects of smoking on 25 (OH) D levels in male smokers.

Material and Method

The design of the present study was a cross-sectional study to determine vitamin D status and cotinine levels among young smokers. This research was approved by the Ethics Committee on Human Rights related to Human Experimentation, Mahidol University, No. MUPH2009-003.

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Subjects

The population of the present study was 67 males who were 25-44 years old and smoked every day. Participants who had hypertension, diabetes, kidney and liver disease, or who had reported they were an ex-smoker or non smoker were excluded.

Urine and blood collection

Spot urine samples were collected in polyethylene bottles. The urine specimens (20 ml) were collected and stored at -20°C. The venous blood (3 ml) was also collected and sent to laboratory to determine 25 (OH) D by chemiluminescent immunoassay. Participants were interviewed for general characteristics, smoking status, health status and behavior.

Chemicals and reagents

Cotinine and 2-phenylimidazole (Internal Standard) were purchased from Sigma (St. Louis, MO, USA). Dichloromethane and potassium hydroxide were purchased from Merck (Darmstadt, Germany). All other chemicals and solvents were HPLC grade. A commercial chemiluminescent immunoassay (LIAISON® 25 OH Vitamin D total) was used to determine the 25 (OH) D in serum.

Instrumentation

High performance liquid chromatography was conducted (Agilent 1100 series, G1357 AA) using a photo diode array detector (HP G1315A DAD-detector) equipped with a platinum column C18, 100A, 5 µm, 150 mm x 4.6 mm ID (Altech) at 25°C and a guard column C18 (Part No. WAT044380, Waters). The mobile phase was a mixture of aqueous phase: methanol: acetonitrile (81: 10: 9) at a flow rate of 1.0 ml/min. Absorbance was observed at 260 nm.

Preparation of solutions

Stock standard of cotinine solution (1 mg/ml) was prepared by dissolving 0.1 g of cotinine in 100 ml methanol. The solution was stored at -20°C.

Internal standard solution (1 mg/ml) was prepared by dissolving 0.1 g of 2-phenylimidazole in 100 ml methanol. The solution was stored at -20°C. Diluting internal standard solution (1 mg/ml) with methanol to give a concentration of 20 µg/ml before use.

The aqueous phase contained 0.372 g of sodium octanesulfonate, 5.15 g of potassium dihydrogen phosphate and 7.92 g of citric acid with 5 ml of

triethylamine before completing to 800 ml with distilled water.

Determination of cotinine in urine

The determination of urinary cotinine was modified from the present study of Ceppia et al⁽⁵⁾. Thawed urines were centrifuged in order to avoid impurities. A total of 5 ml of urine and 125 µl of internal standard (2 phenylimidazole, 20 µg/ml) were added to 20 ml screw capped glass tubes. After the addition of 1 ml of 5 M potassium hydroxide and 5 ml of dichloromethane, the tubes were sealed hermetically, mixed for 2 min and centrifuged at 2,500 rpm for 15 min. The aqueous layer was discarded and 3 ml of the organic phase was collected and protected from light and was evaporated to dryness under a gentle stream of nitrogen at ambient temperature. The residues were dissolved in 300 µl of mobile phase and an aliquot of 30 µl was injected into the HPLC system via an automatic sampler.

Calibration curves of urinary cotinine by HPLC

The working cotinine standard solutions (0.05-3.0 µg/ml urine) were prepared and were analyzed for 3 replications. The calibration curves (0.05, 0.25, 0.75, 1.5, 2.0 and 3 µg/ml urine) were obtained by plotting the peak area ratios of cotinine/internal standard versus the cotinine concentrations.

Accuracy and precision of the method

The known concentrations of cotinine in normal urine were prepared at 0.15, 0.75 and 2 µg/ml urine. They were analyzed in the same manner as the urine samples. The accuracy and precision were calculated for between assays and presented as percent recovery and relative standard deviation (% RSD).

Detection limit of the method

The five concentrations of cotinine standard ranged from 0.01 to 0.05 µg/ml urine were prepared and analyzed. The detection limit was calculated following the National Institute of Occupational Safety and Health, NIOSH⁽⁸⁾.

Determination of urinary creatinine

The urinary creatinine concentrations were determined by Roche/Integra 700/800.

Determination of serum 25 hydroxyvitamin D concentration

Serum concentrations of 25 (OH) D were determined by a commercial chemiluminescent immuno

assay (LIAISON® 25 OH Vitamin D total).

Statistical analysis

The general characteristic of all data were presented as percentage, mean and standard deviation. Pearson correlation coefficient was used for testing the relationship between serum 25 (OH) D concentration and BMI. The relationship between urinary cotinine and the number of cigarettes smoked was determined by Spearman's rho correlation. The comparison of average serum 25 hydroxy vitamin D, urinary cotinine concentrations and number of cigarettes smoked between dairy cooperative smokers and other smokers were compared by Independent t-test.

Results

Chromatograms of urinary cotinine and 2-phenylimidazole solution (internal standard)

The chromatogram of cotinine and 2-phenylimidazole (internal standard) was presented in Fig. 1. The retention times of cotinine and 2-phenylimidazole were 3.369 and 5.531 min, respectively.

Calibration curves of cotinine in urine

The calibration curve of cotinine was linear over the concentration range from 0.05 to 3 µg/ml. The calibration curve showed a linear relationship between relative peak area ratios against cotinine concentrations as $y = 0.7002x + 0.0099$, $r = 0.9998$.

Accuracy and precision of the method

The percent recoveries of the method for analysis of cotinine concentrations of 0.15-2.0 µg/ml were 93.03% to 102.54% and the percent relative standard deviation (% RSD) was in the range of 4.28% to 11.45%.

The detection limit of the method

The detection limit of the method for analysis

of cotinine concentrations was 0.02 µg/ml.

General characteristics of smokers

The average age of 67 male smokers was 32.6 years old. A majority of subjects (82.1%) were dark skin. There were 43.3% of subjects with normal weight. The average number of cigarettes smoked per day was 7.97, ranged from 1 to 20 and the subjects who smoked more than 10 cigarettes per day were 43.3%. The average duration of smoking was 14.24 years. The smokers were categorized into two groups, the dairy co-operative smokers and other smokers. The characteristics of the two groups are presented in Table 1.

The urinary cotinine concentrations of smokers

The urinary cotinine concentrations of smokers are shown in Table 2, with average of 1,680.5 µg/g creatinine, ranged from 63.20 to 7,182.40 µg/g creatinine.

The relationship between urinary cotinine concentrations and the number of cigarettes smoked

The urinary cotinine concentration was significantly associated with the number of cigarettes smoked ($p < 0.001$) as shown in Table 3.

The serum 25 hydroxyvitamin D concentrations of smokers

The average serum 25 (OH) D of smokers in the present study was 25.34 ± 7.13 ng/ml. The guideline for optimal serum 25 (OH) D levels in young and older adult was set at ≥ 30 ng/ml to prevent osteoporosis, rickets, bone fracture and myopathy⁽⁹⁻¹³⁾. In the present study, the serum 25 (OH) D concentration in most subjects (79.1%) were well below the 30 ng/ml threshold value. Only 21.9% of subjects had serum 25 (OH) D compliant with the threshold value (≥ 30 ng/ml).

The relationship between serum 25 hydroxyvitamin D concentrations and BMI

The serum 25 (OH) D concentrations were significantly negative correlated with BMI ($p < 0.05$) as shown in Table 4.

The comparison of average serum 25(OH)D concentrations between dairy cooperative smokers and other smokers

The average number of cigarettes smoked per day and urinary cotinine concentrations was not

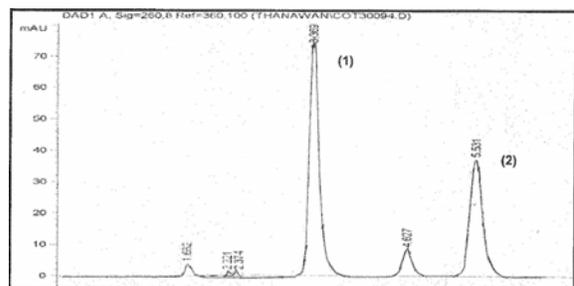


Fig. 1 The chromatogram of standard cotinine (1) and 2-phenylimidazole (2)

Table 1. The general characteristics of dairy co-operative smokers and other smokers (n = 67)

Characteristics	Dairy co-operative smokers (n = 28) Number	Other smokers (n = 39) Number
Age (years)		
25-34	15	29
35-44	13	10
BMI (kg/m ²)		
< 18.5	1	5
18.5-22.9	9	20
23-24.9	9	6
≥ 25	9	8
Color of skin		
White	3	9
Dark	25	30
Number of cigarettes smoked (per day)		
< 10	14	24
≥ 10	14	15
Duration of smoking (years)		
≤ 10	8	11
11-20	16	24
21-30	4	4

Table 2. The urinary cotinine concentration of active smokers

Cotinine concentration (µg/g Cr)	Number (n = 67)	Percentages
≤ 1,000	29	43.3
1,001-2,000	18	26.9
≥ 2,001	20	29.9
Mean ± SD = 1,680.51 ± 1,572.96 (Min = 63, Max = 7,182.40)		

Table 3. The relationship between urinary cotinine of smokers and the number of cigarettes smoked

Parameters	Mean	SD	Spearman's rho	p-value
Cotinine (µg/g Cr)	1,680.51	1,572.96	0.429	< 0.001
No. of cigarettes smoked/day	7.97	4.592		

significantly different in the dairy co-operative smokers and other smokers as shown in Table 5 at p-values of 0.221 and 0.256, respectively. The average serum 25 (OH)D levels were significantly different at $p < 0.001$ in the dairy co-operative smokers and other smokers. When the serum 25 (OH)D levels of individual smokers in the dairy cooperative smokers and other smokers were compared with the 30 ng/ml threshold value, it was found that 42.86% and 10.26% of the dairy cooperative smokers and other smokers had serum 25 (OH)D levels of ≥ 30 ng/ml to prevent osteoporosis,

rickets, bone fracture and myopathy⁽⁹⁻¹³⁾.

Discussion

Analytical method

The procedure for the determination of urinary cotinine in the present study was modified from the present study of Ceppa et al⁽⁷⁾. They used a C8 symmetry cartridge column, whereas the present study used a C18 column. The run time for the analysis of urinary cotinine in the present study was 10 minutes, which was less than the previous study (16 minutes)⁽⁷⁾.

Table 4. The relationship between serum 25 hydroxyvitamin D concentrations and BMI

Parameters	Mean	SD	Pearson Correlation	p-value
25 (OH) D (ng/ml)	25.34	7.13	-0.242	0.049
BMI (kg/m ²)	22.88	3.53		

Table 5. The comparison of average serum 25 (OH) D, urinary cotinine concentrations and number of cigarettes smoked between dairy co-operative smokers and other smokers

Parameters	Dairy co-operative smokers (n = 28)	Other smokers (n = 39)	p-value
	Mean ± SD	Mean ± SD	
Number of cigarettes smoked per day	8.79 ± 6.23	7.38 ± 2.86	0.221
Cotinine concentration (µg/g creatinine)	1,421.42 ± 1493.31	1,866.52 ± 1,621.08	0.256
25 (OH) D (ng/ml)	29.09 ± 6.38	22.65 ± 6.45	< 0.001

The lowest cotinine concentration detected in the present study was 0.02 µg/ml whereas the previous studies reported the minimum detectable concentration of urinary cotinine was 0.005 µg/ml^(7,14).

General characteristics of smokers

This research was performed on active smokers during the rainy season which has a lower level of sunlight. The active smokers were divided into 2 groups, dairy co-operative smokers and other smokers. The average age of dairy co-operative smokers and other smokers was 34 and 32 years old, respectively; who were considered as young adult. The amount of bone resorbed and bone formed is balance in young people. There were 32.1% of dairy co-operative smokers and 23.5% of other smokers were obese. The majority of dairy co-operative smokers (89.3%) and other smokers (76.9%) were dark skin. The dark skin was caused by the intensity of melanin in the skin, which can protect the skin from sunlight and resulted to reduce the synthesis of vitamin D⁽¹⁵⁾.

The relationship between serum 25 (OH) D concentrations and BMI

The serum 25 (OH) D concentrations were significantly inverse correlated with BMI ($p < 0.05$). It is because of parent compounds of 7-decholesterol were trapped in adipose tissue of those with high BMI which can lead to low levels of vitamin D synthesis. The authors' finding agrees with the previous study that healthy middle aged having BMI more than 30 kg/

m² had low vitamin D status⁽¹⁶⁾.

The relationship between urinary cotinine concentrations and the number of cigarettes smoked

The results showed that urinary cotinine concentration was significantly correlated with the number of cigarettes smoked ($p < 0.001$) which coincided with the previous study⁽⁷⁾.

The comparison of average serum 25 (OH) D concentrations between dairy cooperative smokers and other smokers

The serum 25 (OH) D concentrations in dairy co-operative smokers were significantly higher than the other smokers ($p < 0.05$). In addition, the dairy co-operative smokers had a higher percentage of subjects having the serum 25 (OH) D concentrations ≥ 30 ng/ml than the other smokers, while urinary cotinine levels and the number of cigarettes smoked in these two groups were not significantly different. This is probably because the dairy co-operative smokers consumed weekly dietary vitamin D at a higher level than the other smokers. The previous study⁽¹⁶⁾ indicated that milk intake was a source of vitamin D in the diet. It can be seen that the dairy co-operative smokers had serum 25 (OH) D concentrations of 29.09 ng/ml which was close to 30 ng/ml for non smokers due to dairy intake.

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Potential conflicts of interest

None.

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ระดับโคตินีนในปัสสาวะและ 25 ไฮดรอกซีวิตามินดีในซีรัมในผู้ชายที่สูบบุหรี่

ธนวรรณ บัวเจริญ, พรพิมล กองทิพย์, ประพิน ธารภูผาสยาม, สุทธินันท์ ฉันท์ธนกุล, ดุสิต สุจิรัตต์

วัตถุประสงค์: เพื่อวิเคราะห์ปริมาณโคตินีนในปัสสาวะและ 25 ไฮดรอกซีวิตามินดีในซีรัมของผู้ชายที่สูบบุหรี่จำนวน 67 คน

วัสดุและวิธีการ: ทำการวิเคราะห์ตัวอย่างปัสสาวะและเลือดเพื่อหาความเข้มข้นของโคตินีนในปัสสาวะและ 25 ไฮดรอกซีวิตามินดีในซีรัมด้วยเครื่องไฮเพอร์ฟอร์แมนซ์ ลิกวิดโครมาโตกราฟี (HPLC) และเคมีลูมิเนสเซนซ์ อิมมูโนแอสเสย์ ตามลำดับ ค่าความถูกต้อง ความแม่นยำและค่าต่ำสุดที่ตรวจวัดได้ของวิธี HPLC ได้นำมาทดสอบด้วย

ผลการศึกษา: ค่าต่ำสุดของโคตินีนในปัสสาวะที่วิเคราะห์ได้เป็น 0.02 ไมโครกรัมต่อมิลลิลิตร ค่าการวิเคราะห์ กลับคืนของโคตินีนความเข้มข้น 0.15-2.0 ไมโครกรัมต่อมิลลิลิตร มีค่ามากกว่าร้อยละ 95 คนสูบบุหรี่เพียงร้อยละ 23.9 ที่มีระดับของ 25 ไฮดรอกซีวิตามินดี อยู่ในเกณฑ์เพียงพอในซีรัมอย่างน้อย 30 นาโนกรัมต่อมิลลิลิตร กลุ่มคน สูบบุหรี่แบ่งออกเป็นพนักงานสหกรณ์โคนมและพนักงานอื่นๆ ค่าเฉลี่ยความเข้มข้นของโคตินีนในปัสสาวะเป็น 1,421.42 และ 1,866.52 ไมโครกรัมต่อกรัม ครีเอทีนิน ขณะที่ค่าเฉลี่ย 25 ไฮดรอกซีวิตามินดีในซีรัมเป็น 29.09 และ 22.65 นาโนกรัมต่อมิลลิลิตรในพนักงานสหกรณ์โคนมและพนักงานอื่นๆ ที่สูบบุหรี่ตามลำดับ

สรุป: พนักงานสหกรณ์โคนมและพนักงานอื่นๆ ที่สูบบุหรี่ ร้อยละ 42.86 และ 10.26 มีระดับ 25 ไฮดรอกซีวิตามินดี ในซีรัมเพียงพอที่จะป้องกันโรคกระดูกพรุน
